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# Mason–Pfizer monkey virus Gag proteins interact with the human sumo conjugating enzyme, hUbc9

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**Abstract**

Retroviral Gag proteins function during early and late stages of the viral life cycle. To gain additional insight into the cellular requirements for viral replication, a two-hybrid screen was used to identify cellular proteins that interact with the Mason–Pfizer monkey virus Gag protein. One of the cellular proteins found was identified as hUbc9, a nuclear pore-associated E2 SUMO conjugating enzyme. In vitro protein interaction assays verified the association and mapped the interaction domain to the CA protein. In vivo, hUbc9 and Gag colocalized in the cytoplasm as discrete foci near the nuclear membrane. In addition, overexpression of hUbc9 in cells caused a fraction of Gag to colocalize with hUbc9 in the nucleus. These experiments demonstrate that hUbc9 and Gag interact in cells, strengthen the hypothesis that Gag proteins transiently associate with the nuclear compartment during viral replication, and suggest that hUbc9 plays a role in this process.



*Keywords:* Mason–Pfizer monkey virus; Retroviridae; Gag; Capsid; Virus assembly; Ubc9; Yeast two-hybrid screen; COS-1 cells

## Introduction

Retroviral Gag proteins play critical roles during early and late stages of the viral life cycle. It is clear from numerous studies that Gag proteins drive virus assembly and can do so when expressed in the absence of all other viral components. Thus, Gag contains the necessary structural information for intracellular transport, assembly of the immature capsid shell, plasma membrane binding, and budding. Gag proteins are also responsible for packaging the unspliced viral RNA, the *pol* gene encoded reverse transcriptase (RT) and integrase (IN) enzymes and the *env* gene encoded viral glycoproteins. Either during or shortly after budding, Gag is cleaved by the viral protease into the internal structural and enzymatic proteins of the mature infectious virion. These include MA (matrix), CA (capsid), and NC (nucleocapsid). For many retroviruses, additional Gag-derived polypeptides are found in the mature virion but their precise locations and functions within the mature virion are not fully understood.

Gag proteins are also thought to function during entry into a new cell. They have been found to be components of the preintegration complex (PIC) and many mutations in the *gag* genes of various retroviruses have no obvious effect on assembly but rather block replication during early stages of infection (Alin and Goff, 1996; Crawford and Goff, 1984; Parent et al., 1996; Scheifele et al., 2002; Yasuda and Hunter, 1998; Yu et al., 1992). It is, therefore, reasonable to suspect that Gag proteins play active roles in the processes of uncoating, reverse transcription, transportation of the PIC to the nuclear compartment, or integration.

Mason–Pfizer monkey virus (MPMV) is the prototypical betaretrovirus. As with other betaretroviruses, the MPMV Gag polyproteins assemble into immature procapsids in the cytoplasm, near the nuclear membrane. Once assembled, the procapsids are actively transported to the plasma membrane from which they bud. Unlike all other retroviruses that assemble and bud concomitantly at the plasma mem-

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brane, the processes of betaretrovirus capsid assembly, cytoplasmic transport, membrane binding, and budding are separate events. Indeed many mutations in MPMV Gag have been found which specifically block each of these morphogenic events (reviewed in Hunter, 1994; Sakalian and Hunter, 1999; Weldon and Hunter, 1996). Other point mutations have been found which convert the MPMV morphogenic pathway to one that resembles other retroviruses such as HIV-1 and RSV (Rhee and Hunter, 1991). For these reasons, MPMV serves as an excellent model for determining the many molecular events during retrovirus replication.

During virus maturation, the MPMV Gag precursor polyproteins are cleaved by the viral protease into the individual structural proteins. These include MA, pp24, p12, CA, NC, and p4. The phosphoprotein pp24 is further cleaved into pp18 and pp16. The function of pp18 protein is not known but appears to be important for early replicative events. pp16 contains the late budding motif (L) and is required for particle release (Yasuda and Hunter, 1998; Yasuda et al., 2002). The p12 protein is also important during assembly, where it appears to provide a scaffolding function for efficient assembly (Sakalian and Hunter, 1999; Sommerfelt et al., 1992).

It has become evident that Gag proteins function during virus replication via interactions with host cell components (Weldon et al., 1998). KIF4, a member of the kinesin family of microtubule-associated motor proteins, has been found to interact with the Gag proteins of HIV-1, MLV, and MPMV in two-hybrid assays (Kim et al., 1998). These findings, along with the observations that actin and other cytoskeletal proteins are packaged into retrovirus particles (Ott et al., 1996), have led to the hypothesis that Gag proteins are transported through the cytoplasm along the cytoskeletal network. Virion release requires functional interactions between Gag L domains and cellular proteins involved in ubiquitin ligation, NEDD4 and BUL1, or in late endosomal trafficking, TSG101 and Vsp4 (Demirov et al., 2002; Garrus et al., 2001; Kikonyogo et al., 2001; Martin-Serrano et al., 2001; Myers and Allen, 2002; Pornillos et al., 2002; Strack et al., 2000; VerPlank et al., 2001; Yasuda et al., 2002). Chaperonins and chaperonin-associated proteins, TriC, Hsp70, and Ubp, have been found to interact with MPMV and HIV-1 Gag proteins and it has been suggested that these cellular proteins assist in folding Gag proteins into a conformation(s) required for capsid assembly (Callahan et al., 1998; Gurer et al., 2002; Handley et al., 2001; Hong et al., 2001). HEED, the human homolog of mouse eed (Peytavi et al., 1999), translation elongation factor 1-alpha (Cimarelli and Luban, 1999), HO3, a putative tRNA synthetase (Lama and Trono, 1998), and hIF2, a human homolog of bacterial translation initiation factor 2 are known to interact with the HIV MA protein and provide essential replicative functions. However, the roles these proteins play during viral replication are not known.

Gag-interacting cellular proteins are also thought to be essential for virus replication during the early stages of the viral life cycle. Cyclophilin A, a prolyl isomerase thought to function as a chaperonin, is incorporated into HIV-1 particles via interactions with Gag. This incorporation enhances viral infectivity (Braaten et al., 1996; Luban et al., 1993). The antiviral *Fv1* gene product interacts with the CA protein of MLV and abrogates integration at a postentry step (Goff, 1996; Rosenberg and Jolicoeur, 1997). It has also been suggested that the Gag-interacting nuclear shuttling proteins, Van and CRM-1, regulate nuclear import of the HIV-1 and RSV PICs (Bacharach et al., 2000; Gupta et al., 2000; Scheifele et al., 2002). It is likely that many other cellular proteins interact with Gag proteins and are involved in retrovirus replication.

To gain more insight into the cellular requirements for viral replication, we utilized a two-hybrid screen to search for cellular proteins that interact with the MPMV Gag protein. We show that MPMV Gag specifically interacts with hUbc9, a nuclear pore-associated, E2 SUMO conjugating enzyme. Sumoylation is the process of covalently attaching a SUMO moiety to a target protein (Melchior, 2000; Wilson and Rangasamy, 2001). SUMO is small protein (101 amino acids) with amino acid sequence similarity to ubiquitin (Lapenta et al., 1997). While ubiquitination and sumoylation are biochemically analogous processes, they are different enzymatic pathways leading to alternative biological roles. While polyubiquitination targets proteins for degradation, sumoylation does not. Instead, Ubc9, through its SUMO conjugase activity, (i) controls intracellular trafficking of substrate proteins to the nuclear compartment (Bhaskar et al., 2000; Duprez et al., 1999; Rangasamy et al., 2000) or the mitotic spindle (Joseph et al., 2002; Saitoh et al., 1997, 1998); (ii) facilitates protein–protein interactions (Subramanian et al., 2003); or (iii) facilitates protein function (Desterro et al., 1998; Giorgino et al., 2000; Rodriguez et al., 1999; Tashiro et al., 1997). Other reports indicate that Ubc9 may have the capacity to function independently of its enzymatic activity. Vsx-1, for example, is a paired-like homeobox transcription factor that requires an interaction with Ubc9 for nuclear localization yet is not sumoylated (Kurtzman and Schechter, 2001).

Here we describe the interaction of hUbc9 with the MPMV Gag precursor polyprotein, Pr78, and the Gag-cleavage product CA. We demonstrate that these interactions take place in vitro using GST pull-down and coimmune precipitation assays and show that hUbc9 and Gag proteins clearly colocalize in the cytoplasm as discrete foci near the nuclear membrane. In addition, we found that overexpression of hUbc9 in cells caused a fraction of Gag to colocalize with hUbc9 in the nucleus. These experiments demonstrate that hUbc9 and Gag interact in cells and raise the possibility that Gag proteins, through interactions with hUbc9, transiently associate with the nuclear compartment during viral replication.

## Results

### *Identification of hUbc9 as a MPMV Gag-interacting protein*

To identify MPMV Gag-interacting proteins, a Gal4-based, yeast two-hybrid system programmed with the full-length Gag as a bait was used to screen a HeLa cDNA library. Yeast were transformed with the pAS.Gag bait plasmid and the cDNA library expression plasmids. A total of $2.5 \times 10^7$ clones were screened. Of these clones, 348 were initially found to activate both reporter genes, *HIS3* and *Lac*Z. From the initial candidate clones obtained, 212 clones were reconfirmed after three rounds of replating on selective media containing increased amounts of 3-aminotriazole 3-AT (15 mM) to block leaking *HIS3* expression. Candidate library plasmids were retransformed into yeast either by itself, with the empty bait plasmid, pSD2.1, or into yeast containing pAS.Gag. Twenty-five clones reproducibly activated the *lac*Z and *HIS3* reporter genes only in the presence of the pAS.Gag. DNA sequence analysis of the 25 prey plasmids revealed that 15 of the clones contained a nearly identical 5′ untranslated region (average length of 125 bp), a 3′ untranslated region of 564 bp, and an open reading frame of 474 bp encoding a 158 amino acid protein with an estimated molecular weight of 18 kDa. Comparison of the open reading frame sequence with sequences in GenBank databases demonstrated 100% identity to hUbc9, a human homologue of the E2 Sumo-conjugating enzyme Ubc9 in yeast (Hateboer et al., 1996; Wright et al., 1996). In addition, 9 of the 25 initial clones contained a common open reading frame identical to Tsg101, which has been previously shown to interact with the late budding domains of HIV-1 and HIV-2 Gag and the ebola virus p40 protein and facilitate virus egress (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; Myers and Allen, 2002; Pornillos et al., 2002; VerPlank et al., 2001). The hUbc9 clone, G10B, was chosen for further studies because it contained minimal upstream (116 bp) and downstream (553 bp) noncoding regions and a complete, non-permutated coding region. As shown in Table 1, expression of Ubc9–Gal4AD fusion proteins in yeast activated the $\beta$-galactosidase reporter gene in the presence of MPMV Gag and p53 but not in yeast harboring either the empty bait plasmid pAS2.1 or a plasmid encoding Gal4BD–human lamin C. The observation that coexpression of Ubc9 and p53 activated the reporter gene was expected because these two proteins are known to interact (Lin et al., 2002; Rodriguez et al., 1999; Shen et al., 1996).

Table 1
Gag–Ubc9 interactions in the yeast two-hybrid system

| Bait protein (plasmid) | Prey protein (plasmid) | $\beta$-Galactosidase assay | |
|---|---|---|---|
| | | Filter[a] | Liquid |
| Empty vector (pAS2.1) | Empty vector (pACT2) | − | 0.39 ± 0.04 |
| Gag (pASGag) | Empty vector | − | 0.12 ± 0.01 |
| Empty vector | Ubc9 (pG10B) | − | 1.13 ± 0.66 |
| p53 (pVA3) | T Ag (pTD1) | ++ | 22.83 ± 1.00 |
| Gag | Gag (pMPAD) | ++ | 17.12 ± 1.28 |
| Gag | Ubc9 | + | 11.35 ± 1.18 |
| p53 | Ubc9 | + | 8.74 ± 0.6 |
| Lamin C | Ubc9 | − | n.d. |

*Note.* Three independent cotransformations were tested for $\beta$-galactosidase activity (filter and liquid assays). Each bait protein was expressed in yeast as C-terminal fusions proteins with the Ga14 DNA-binding domain.
[a] $\beta$-gal filter assays are reported as −, no visible color after 18 h incubation; +, visible blue color after 4 h incubation; ++, visible blue color after 2 h incubation. n.d., not done.

### *MPMV Gag–hUbc9 interactions*

The results of the yeast two-hybrid experiments were verified by testing whether hUbc9 and Gag interact in vitro. For this, GST pull-down and coimmune precipitation experiments were performed using hUbc9–GST fusion proteins and MPMV Gag proteins derived from the following several different sources: (1) in vitro transcription/translation reactions: (2) cell lysates; and (3) detergent-solubilized virions.

There are several published reports that describe the use of GST fusion proteins to pull-down Gag proteins from different retroviruses (Bacharach et al., 2000; Callahan et al., 1998). However, we found in preliminary experiments that MPMV Gag bound to GST just as efficiently as it did to hUbc9–GST in standard "GST pull-down" buffers. To prevent these nonspecific interactions, equal molar-amounts of GST and hUbc9–GST fusion proteins bound to glutathione–Sepharose beads (12 and 17 $\mu$g, respectively) were incubated with BSA for 15 min and then incubated for 8 h at 4°C with Pr78 translated in vitro from pET.M100A (Sakalian and Hunter, 1999). After washing the beads, proteins bound to the beads were eluted and analyzed by Western blot analysis using antibodies against Pr78. Under these conditions, Pr78 did not bind to either the glutathione sepharose beads or the immobilized GST protein alone. In contrast, Pr78 bound to the hUbc9–GST fusion protein (Fig. 1A). Based on the amount of Pr78 added to these reactions as well as in subsequent repeated experiments (repeated five times, data not shown), hUbc9–GST was estimated to bind approximately 2–3% of the input Pr78. The inability of hUbc9–GST to associate with more than 1–2% of the input Pr78 was not due to limiting amounts of the fusion protein in the assay because hUbc9–GST was added in 100-fold excess to the amount of Pr78. When the nonbound fraction of the initial pull-down was retested for hUbc9–GST binding under the same conditions, essentially the same amount of Pr78 bound to hUbc9–GST (data not shown). These results suggest that the inability of Ubc9–GST to pull down more than 2–3% of the input Pr78 either reflects weak binding or reflects that during dynamic protein conformational changes of Pr78 in solution, only a certain fraction assumes a conformation that is competent for Ubc9 binding.

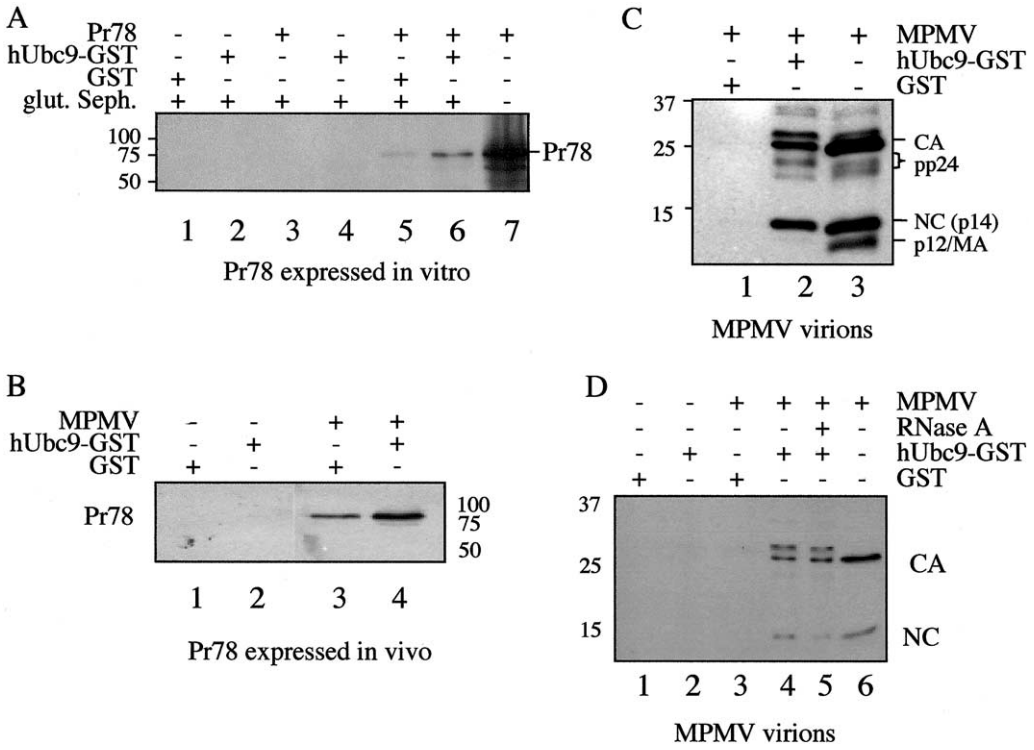


Fig. 1. In vitro interactions between MPMV Gag to Ubc9. (A) Binding of hUbc9-GST to in vitro translated Pr78. GST-bound glutathione–Sepharose beads (lanes 1 and 5), hUbc9-GST-beads (lanes 2, 4, and 6), or glutathione–Sepharose beads only (lane 3) were incubated in buffer only (lanes 1 and 2), a rabbit reticulocyte lysate (lane 4), or rabbit reticulocyte lysates containing pET.M100A, which encodes Pr78 (lanes 3, 5, and 6). Proteins bound to the beads were separated by SDS–PAGE. As a loading control, 1/10 of the amount of in vitro translated Pr78 added to the pull-down assays was loaded directly onto the gel (lane 7). Gag-related proteins were detected by immunoblotting with anti-Pr78 antibodies (A–D) and their positions are indicated. (B) Binding of hUbc9-GST to Pr78 expressed in HeLa cells. Lysates prepared from either untransfected HeLa cells (lanes 1 and 2) or HeLa cells transfected with pSARM4 (lanes 3 and 4) were incubated with either GST beads (lanes 1 and 3) or hUbc9-GST beads (lanes 2 and 4). Bound proteins were analyzed as described for (A). (C) Binding of hUbc9-GST to MPMV virion proteins. Purified MPMV virions were solublized with 0.5% TX-100, incubated with either GST-beads (lanes 1) or hUbc9-GST-beads (lane 2), eluted, and separated by SDS–PAGE. As a loading control, 1/10 of the amount of virions added to the pull-down assays was run directly on SDS–PAGE (lane 3). (D) RNAse A digestion of MPMV-hUbc9-GST protein complexes reduces NC binding. To determine if virion-derived NC interacts indirectly with hUbc9-GST via RNA, Rnase A (5 $\mu$g) was included in the binding reactions (lane 5) as described above. All other lanes were identical to those described for (C) and their contents are indicated.

Nonetheless, these results substantiate the observation that Pr78 interacts with hUbc9.

Further validation of the specific interaction between Pr78 and Ubc9 was obtained with GST pull-down experiments using transfected cell lysates. HeLa cells were mock transfected or transfected with an infectious proviral clone, pSARM4. At 72 h posttransfection, cells were lysed and incubated with hUbc9–GST–Sepharose and GST–Sepharose beads for 8 h at 4°C in the presence of excess BSA. Proteins bound to the beads were eluted and Gag proteins were detected by Western blot analysis using anti-Pr78 antibodies. Again, hUbc9–GST bound significantly more Pr78 (Fig. 1B, lane 4) than did GST-only beads (Fig. 1B, lane 3). These results further confirm that hUbc9 is capable of interacting with Pr78.

The basis for the interactions between hUbc9 and Pr78 was studied further by mapping the region of Pr78 that binds to hUbc9. For these studies, 60 ng of virion-associated, Gag cleavage proteins were extracted from purified, infectious virions and incubated with either equal molar amounts of hUbc9–GST (17 $\mu$g) or GST-only (12 $\mu$g) bound beads. Gag proteins bound to the beads were analyzed by Western blots as before. One-tenth of the amount of virion-derived proteins added to the pull-down reactions was directly loaded on the acrylamide gel as loading and antibody controls (Fig. 1C, lane 3). We found that hUbc9–GST (Fig. 1C, lane 2), but not GST only (lane 1), was able to pull down several Gag-related proteins, CA (p27) and NC (p14). The identity of the 30-kDa protein is assumed to be an intermediate Gag cleavage product. It is important to remember that Pr78 is cleaved by the viral protease during or shortly after particle release into equal molar amounts of MA, pp24, p12, CA, NC, and p4. However, as can be seen in Fig. 1C (lane 3), the antiserum used here did not react well with pp24 from purified virions. We, therefore, could not determine whether the low amount of pp24 observed in the pull-down reactions (Fig. 1C, lane 2) is a reflection of weak interactions between pp24 and hUbc9 or inadequate antibody binding to pp24, or whether pp24 is being pulled down in this assay via interactions with CA and not Ubc9.

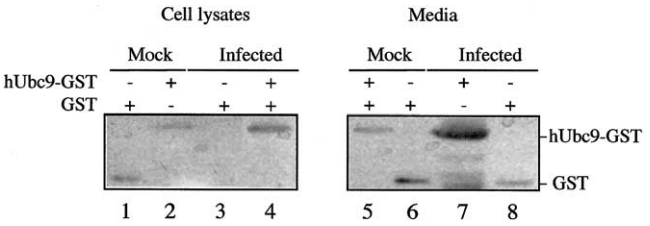


Fig. 2. Coimmunoprecipitation of MPMV Gag and soluble hUbc9-GST. HeLa cells were either mock transfected (lanes 1, 2, 5, and 6) or transfected with 2 μg of wild-type proviral MPMV DNA, pSARM4 (lanes 3, 4, 7, and 8). At 48 h posttransfection, cell-culture media were harvested and cell lysates (lanes 1–4) were prepared. Media samples were centrifuged through a 20% sucrose cushion and the pellets were lysed (lanes 5–8). Samples were incubated with equal molar amounts of soluble GST and hUbc9–GST fusion proteins for 8 h at 4°C. Proteins were collected by immunoprecipitation using polyclonal antibodies to Pr78. Immune precipitates were separated by electrophoreses and analyzed by immunoblotting with HRP-conjugated anti-GST antibodies. Positions of Ubc9-GST and GST are indicated on the right.

Nonetheless, these results suggest that either Pr78 contains multiple Ubc9-binding sites or that one or more of these Gag-cleavage products indirectly interacts with the hUbc9-GST Sepharose beads.

Because NC is an RNA-binding protein, we considered the possibility that NC might associate with the hUbc9–GST beads via RNA and not through direct protein–protein interactions. To test this hypothesis, hUbc9–GST (Fig. 1D, lanes 2, 4, and 5) and GST alone (lanes 1 and 3) -bound glutathione–Sepharose beads were incubated with virion-derived Gag proteins in the presence or absence of exogenously added RNase A. Gag proteins bound to the resins after RNA digestion was analyzed by immunoblotting as before (Figs. 1A–C). Again, CA and NC proteins specifically bound to the hUbc9–GST fusion protein in the absence of RNase A (Fig. 1D, lane 4). As expected, the addition of RNase A did not change the amount of CA bound to hUbc9. In contrast, the amount of NC bound was reduced in the presence of RNase A (Fig. 1D, lane 5). These results suggest that the majority of NC binds to the beads via RNA and not directly to Ubc9. The nature of the interactions observed between hUbc9 and CA and pp24 (i.e., direct or indirect binding) is currently being investigated.

Further verification of Ubc9–Gag interactions was obtained by coimmunoprecipitation experiments. To this end, cell lysates and solubilized virions from mock-transfected HeLa cells and HeLa cells transfected with pSARM4 were incubated with equal molar amounts of soluble hUbc9–GST or GST alone. Protein complexes were immuno precipitated using anti-Pr78 antibodies and analyzed by immunoblotting with anti-GST antibodies. As shown in Fig. 2, low levels of hUbc9–GST and GST alone bound proteins were detected in untransfected cell lysates (lanes 1 and 2) and media (lanes 3 and 4). However, significantly more hUbc9–GST was precipitated from infected cell lysates (lane 4) and virions (lane 7) than was GST (lanes 3 and 8). We have, therefore, used several different experimental procedures to demonstrate that hUbc9 specifically interacts with MPMV Gag proteins in vitro.

*Ubc9 is not incorporated into MPMV virions*

Many but not all cellular proteins found to interact with Gag proteins are packaged into virions either passively due to their interactions with Gag at the site of assembly (e.g., Tsg101) (Myers and Allen, 2002; Demirov et al., 2002) or actively because they are thought to provide preintegrative functions (e.g., cyclophilin A)(Braaten et al., 1996). We, therefore, examined whether hUbc9 is packaged into virus particles. MPMV virions were purified from chronically infected CMMT cells by isopycnic and subsequent velocity sucrose gradients and then digested with subtilisin to remove any proteins that copurified with but were not contained within virus particles. After digestion, virions were purified through a 20% sucrose cushion and equal amounts of proteins in the pellets were analyzed by immunoblotting using antibodies directed against either Pr78 or hUbc9. Several controls were included in this experiment. Uninfected cell lysates served as antibody controls. The second control was an assay to screen for the presence of Tsg101 in MPMV virions. Since Tsg101 was identified in our two-hybrid screen as a putative MPMV Gag-interaction partner and because it has been detected in purified HIV-1 and 2 virions (Demirov et al., 2002; Myers and Allen, 2002), we expected to find Tsg101 within MPMV virions. As shown in Fig. 3, all of the Gag-cleavage products were found in virions but not in uninfected cells lysates (lanes 1 and 2). The 27-kDa band detected in the untransfected sample is due to inadvertent spill over of the sample during the loading of the gel. As expected, Tsg101 was also found in cell

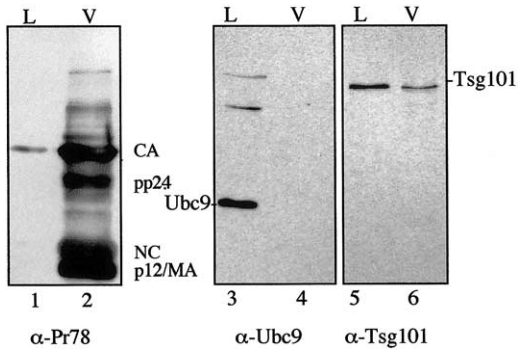


Fig. 3. Tsg101, but not Ubc9, is packaged into MPMV virions. Uninfected cells (L) were lysed in SDS–PAGE sample buffer (lanes 1, 3, and 5). Purified MPMV virions (V) were digested with 10 mg/ml subtilisin for 4 h at room temperature, purified by centrifugation through a 20% sucrose cushion, and solublized in SDS–PAGE sample buffer (lanes 2, 4, and 6). Cellular and viral proteins were analyzed by immunoblotting with polyclonal anti-Pr78 (lanes 1 and 2), monoclonal anti-Ubc9 (lanes 3 and 4), or monoclonal anti-Tsg101 (lanes 5 and 6) antibodies. The locations of the viral proteins (CA, pp24, NC, MA, and p12), hUbc9 and Tsg101 are indicated. The protein present in lane 1 is the result of spillover during the loading of the gel.

lysates and virions (Fig. 3, lanes 5 and 6). In contrast, while hUbc9 was easily observed in uninfected cell lysates (lane 3), it was not packaged into virions at a detectable level (lane 4). Thus, if hUbc9 interacts with Pr78 in cells, it is not packaged into viral particles at significant levels.

*Colocalization of Ubc9 and MPMV Gag proteins*

hUbc9 has been found in several different subcellular compartments. In yeast and *Drosophila*, Ubc9 localizes within the nucleoplasm (Joanisse et al., 1998; Seufert et al., 1995). In mammalian cells, Ubc9 is also found in the nucleus but it is particularly prominent on the cytoplasmic side of the nuclear membrane as a component of the nuclear pore fibrils (Lee et al., 1998; Melchior, 2000; Saitoh et al., 1997, 1998, 2002; Zhang et al., 2002), where it is thought to control intracellular targeting of substrate proteins either directly through protein interactions or indirectly via sumoylation (Kurtzman and Schechter, 2001; Yeh et al., 2000). This subcellular distribution of Ubc9, the ability of Pr78 to form stable complexes with hUbc9, and fact that Pr78 assembles into procapsids on the cytoplasmic side of the nuclear membrane suggested that the Gag and Ubc9 might colocalize. To test this hypothesis, we transfected COS-1 cells with pCMV.Myc-Ubc9 alone or together with pSARM4 and examined the cells by confocal microscopy using antibodies to Gag and to Ubc9. As has been observed for endogenous hUbc9, HA-tagged Ubc9, and Ubc9-GFP fusion proteins (Ahn et al., 2001; Rodriguez et al., 2001; Saitoh et al., 2002), our Myc-tagged hUbc9 localized to the nucleus (excluding the nucleolus) and in the cytoplasm near the nuclear membrane as distinct foci when expressed alone (Figs. 4A and B). Thus, the addition of the Myc tag to the amino-terminal end of hUbc9 did not affect its subcellular localization.

The subcellular distribution of Pr78 was observed in cells expressing normal levels of hUbc9 and in cells overexpressing Myc-Ubc9. In cells with normal levels of hUbc9, Pr78 primarily showed a speckled cytoplasmic staining pattern (Figs. 4D and G). Interestingly, Pr78 was also consistently found in nucleus (albeit at low levels) in these cells. In COS-1 expressing both Pr78 and Myc-Ubc9, the Gag signal closely paralleled the hUbc9 signal; there were very conspicuous areas of colocalization in the cytoplasm near the nuclear membrane as well as within the nucleus (Figs. 4C–E). In fact, we consistently observed more Gag-specific signals in the nuclei of cells overexpressing Myc-hUbc9 than we did in cells expressing normal levels of hUbc9. Finding a similar pattern of Gag-hubc9 colocalization in HeLa cells (Figs. 4D–H) argues that the association of Gag with the nuclear compartment is not restricted to COS-1 cells. Furthermore, the hUbc9-specific targeting of Gag to the nuclear compartment indicates that Gag and hUbc9 interact in cells. Whether our findings are influenced by Ubc9 overexpression is currently being investigated.

There is precedence for Gag proteins to transiently associate with the nuclear compartment. In a recent study, Scheifele et al. showed that the RSV Gag protein localized in the nucleus of cells treated with leptomycin B, which blocks CRM-1-mediated nuclear export (Scheifele et al., 2002). Similarly, HIV-1 Gag proteins were found to accumulate in the nucleus of cells overexpressing either the nuclear shuttling protein, Van, or a HIV-1 Gag protein containing a mutation that disrupts a nuclear export signal in the MA domain (Dupont et al., 1999; Gupta et al., 2000). Nonetheless, the nuclear association of MPMV Gag seen here was surprising because this has never been reported. To further support our observations, we looked for conditions that might accentuate the nuclear association of Gag without overexpressing hUbc9. Because nuclear import and export are ATP-dependent processes, we reasoned that if Gag normally transits through the nuclear compartment, then Gag might accumulate in or around the nucleus of cells under conditions of ATP depletion. To test this hypothesis, MPMV-infected CMMT cells were treated with sodium azide and 2-deoxy-D-glucose for 1 h to deplete intracellular ATP. These conditions have been previously shown to deplete ATP without affecting cell viability (Weldon et al., 1998). The cells were then fixed and the subcellular distribution of Gag was observed by confocal microscopy using a focal *z*-section through the nucleus. As expected, Gag was largely, but not completely, excluded from the nucleus of untreated cells (Figs. 4I and L). In contrast, Gag was observed to localize to the nuclear compartment (both within and around the nucleus) under conditions of ATP depletion (see arrow, Figs. 4K and M). Thus, the nuclear targeting of Gag is not exclusively linked to overexpression of Myc-hUbc9. Furthermore, these results are similar to the findings that the Gag protein of RSV accumulates within the nucleus when the cellular CRM-1 nuclear export pathway was blocked using leptomycin B and with the hypothesis that a fraction of Gag proteins might normally transit through the nuclear compartment during virus replication (Scheifele et al., 2002). Whether MPMV Gag also accumulates within the nucleus during leptomycin B treatment is currently being investigated.

## Discussion

Many of the diverse functions of Gag proteins, from driving virus assembly to assisting in early replicative events, have been well characterized over the last several decades (Craven and Parent, 1996; Freed, 1998; Hunter, 1994; Weldon and Hunter, 1996; Wills and Craven, 1991). In more recent years, many laboratories have focused their efforts on identifying and characterizing host cell components utilized by Gag proteins during the various stages of the virus life cycle. The aim of this study was to gain insight into retrovirus replication through an analysis of virus–host interactions by identifying host cell proteins that interact with MPMV Gag. In this article, we describe the discovery

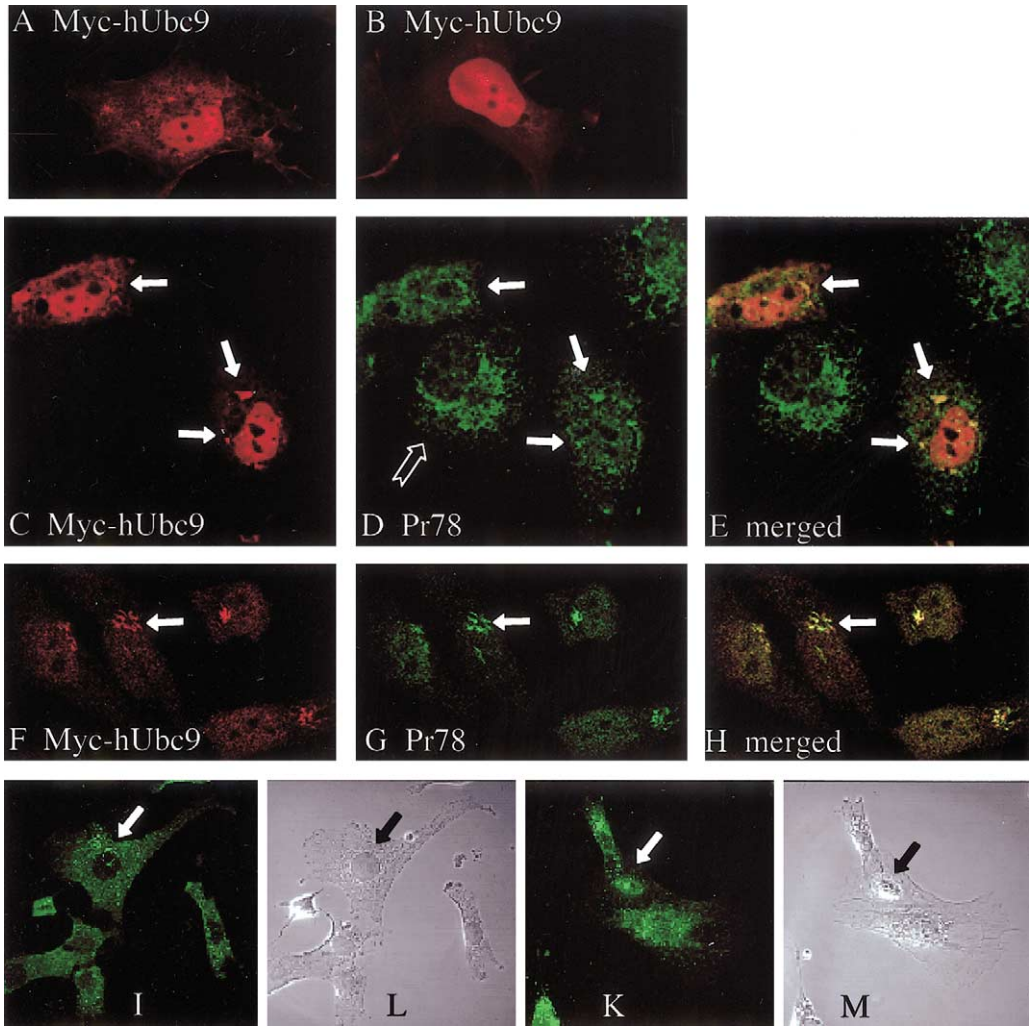


Fig. 4. Subcellular localization of Gag and Myc-tagged Ubc9. (A-E) COS-1 cells were transfected with pCMV. Myc-Ubc9 alone (A and B) or cotransfected with pSHRM15 and pCMV. Myc-hUbc9 (C–E), fixed at 48 h posttransfection, and stained with monoclonal anti-Myc (A, B, C, and F) and polyclonal anti-Pr78 (D and G) antibodies. Polyclonal and monoclonal antibodies were stained with secondary Cy2-conjugated anti-rabbit and Cy5-conjugated anti-mouse antibodies, respectively, and cells were visualized by confocal microscopy. (D–I) HeLa cells were contransfected with pCMV.Myc-Ubc9 and pSHRM15 and prepared in an identical manner but were visualized as a 1-$\mu$m-thick confocal *z*-stack. A representative focal section through the nuclear plane is shown (F–H). Cy2 and Cy5 channels were recorded separately and merged digitally (E and H). (I–M) CMMT cells were left untreated (I and L) or treated with sodium azide and 2-deoxy-D-glucose (K and M) for 1 h to deplete intracellular ATP pools. The subcellular localization of Pr78 (I and K) was detected as described for (A). Phase images of the cells in (I) and (K) show the positions of the nuclei (L and M, respectively).

that hUbc9 interacts specifically with MPMV Gag. The specificity of the interaction observed in yeast was substantiated by several experimental findings. First, purified hUbc9–GST fusion proteins specifically bound Gag precursor polyproteins obtained from in vitro translation reactions and transfected cell lysates. Moreover, purified hUbc9–GST was found to interact with the capsid protein when incubated with solubilized virions. Second, hUbc9 could be coimmunoprecopitated with Gag proteins using anti-Pr78 antibodies. Finally and importantly, coexpression of Myc-hUbc9 and Gag in COS-1 and HeLa cells revealed a reproducible colocalization in the cytoplasm most notably as foci or speckles closely associated with nuclear membrane and to a lesser extent within the nuclear compartment. Whether these cytoplasmic complexes consist solely of Ubc9 and Pr78 is currently being investigated. Nonetheless, these results strongly argue that hUbc9 and MPMV Gag interact in mammalian cells near the nuclear membrane.

In addition to its sumoylating activity, Ubc9 is known to shuttle between the cytoplasm and nucleus and to associate with nuclear pores (Zhang et al., 2002). In doing so, Ubc9 plays a role in intracellular trafficking of substrate proteins to the nuclear compartment, to the kinetochores, and to microtubule organizing centers (Joseph et al., 2002; Kurtzman and Schechter, 2001; Melchior, 2000; Rangasamy et al., 2000; Saitoh et al., 1998; Zhang et al., 2002). Because MPMV Gag is known to assemble in the cytoplasm in the vicinity of the nuclear membrane, we envision several possible functions for hUbc9 during MPMV replication. First, hUbc9 might target a fraction of Pr78 molecules to the

nuclear pore where they bind to unspliced and untranslated viral RNAs as the RNAs exit the nucleus. This Gag–RNA complex could then serve as a nucleation site for procapsid assembly. Such a scenario would explain how Gag proteins and ribosomes compete for the viral RNA for packaging or translation. Second, Ubc9 might target Pr78 into the nucleus where viral RNA recognition might first occur, as suggested by Scheifele et al. (2002). In this scenario, Pr78 would need to contain nuclear import and export signals. Sequences resembling a basic import signal and a leucine-rich export signal are present in MPMV Gag. Whether these are functional nuclear import and export signals is currently being examined. Finally, Ubc9, either directly or indirectly through its sumoylation activity, might be involved in transporting Pr78 (or possibly assembled procapsids) to microtubule organizing centers for eventual transport to the plasma membrane. The fact that MPMV Gag is capable of interacting with the microtubule-associated motor protein, KIF4, in a two-hybrid assay is consistent with this possibility (Kim et al., 1998).

Ubc9 might also play an important role during the early stages of MPMV replication. For example, Ubc9, through its sumoylation activity, might modify Gag proteins of the PIC and as result control targeting of the PIC to the nuclear compartment. Whether Pr78 and/or its cleavage products are sumoylated in infected cells is currently being investigated. Alternatively, Ubc9 might target the PIC to mitotic spindles via sumoylation much in the same way as nuclear targeting of RanGAP1 requires sumoylation (Joseph et al., 2002). In this model, the PIC would remain associated with the chromatin during nuclear reassembly and thus be poised for proviral integration. The models presented here are not mutually exclusive; Ubc9 might play dual roles in assembly and at some postentry step. If Ubc9 is indeed essential for retroviral replication, it would present a target for the design of rational antiviral therapeutics.

## Materials and methods

### *Cell lines, bacteria, and yeast strains*

COS-1 and HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. CMMT cells, which produce infectious M-PMV, were cultured in RPMI 1640 supplemented with 5% tryptose phosphate broth and 10% fetal bovine serum. For ATP depletion studies, CMMT cells were treated with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose in glucose-free RPMI 1640 as previously described (Weldon et al., 1998b). *Escherichia coli* LeuB-HB101 strain was used for selection of the pGAD424-plasmids (prey plasmids) isolated from yeast. *E. coli* BL21 (DE3) strain was used for recombinant GST–fusion protein expression. Plasmid propagation was performed in *E. coli* DH1, DH5$\alpha$, or JM109 strains. *Saccharomyces cerevisiae* Y187, Y190, and CG1945 strains (Clontech) containing the Gal4 promoter-dependent selectable markers *Lac*Z and *HIS3* (stains Y190 and CG1945 only) were used for the yeast two-hybrid experiments.

### *DNAs*

The bacterial expression plasmid pET.M100A encodes a carboxy-terminal His-tagged MPMV Gag protein (Sakalian and Hunter, 1999). Plasmids pSHRM15 and pSARM4 are infectious molecular clones of wild-type MPMV (kind gift from E. Hunter, University of Alabama, Birmingham, AL).

### *Antibodies*

Polyclonal anti-Pr78 has previously been described (Sakalian et al., 1996; Weldon et al., 1998b). Polyclonal and monoclonal antibodies to hUbc9 and Tsg101 were obtained from either Signal Transduction Laboratories or Santa Cruz Biotechnology. HRP-conjugated GST antibodies and HRP-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Amersham Pharmacia. Cy2 and Cy5-conjugated anti-rabbit and anti-mouse antibodies were obtained from Jackson Immunoresearch Laboratories.

### *Two-hybrid screening*

For the yeast two-hybrid screen, the MPMV *gag* gene was inserted into the bait vector pAS2.1 (Clontech), which fuses the GAL4 DNA-binding domain to the amino-terminal end of the Gag protein. For this, the NC-p4 coding region contained within an *Nco*I-*Xho*I fragment of pETM100A was subcloned into the *Nco*I-*Sa*II sites in pAS2.1 (Clontech) to create pAS.NCp4. To subclone the MA-CA region *gag*, the *Nco*I-*Nco*I fragment of pET.M100A was subcloned into the *Nco*I site of pAS.NCp4 to create pAS.Gag. The MPMV *gag* gene was similarly cloned into the yeast Gal4 activation domain plasmid, pGADGH, to create pMPAD. A commercially available HeLa cDNA library cloned into pGADGH (Clontech) was used to screen for cDNAs that encode potential MPMV Gag-interacting cellular proteins. This library had a complexity of $7 \times 10^6$ independent clones.

The Matchmaker 2 system (Clontech) was used for the yeast two-hybrid screen according to manufacturer's suggestions. *S. cerevisiae* CG1945 strain was transformed with pAS.Gag and selected on minimal medium lacking tryptophan. Gal4–Gag fusion protein expression in yeast was confirmed by immunoblot analysis using anti-Gag antibodies (data not shown). Cells were subsequently transformed with the cDNA library and selected on minimal media lacking leucine and tryptophan. Duplicate samples were plated on minimal media lacking leucine, tryptophan, and histidine and supplemented with $\beta$-galactosidase and 5 mM 3AT to select for potential Gag-interacting partners. A total of $2.5 \times 10^7$ transformants were screened for reporter gene activities. Of the resulting 348 colonies that were $His3^+$ and

$\beta$-gal$^+$ (filter assays), 25 were selected that showed dependence on the Gal4–Gag fusion protein for activation of the reporter genes. After three successive platings on selective media to ensure the isolation of pure cultures, plasmid DNAs were isolated and used to transform *E. coli* LeuB-BH101. Transformed bacteria were plated on minimal media lacking leucine for selection of the pGADGH-cDNA plasmids. Plasmid DNAs were isolated from these bacteria and retransformed into *S. cerevisiae* (either Y187 strain for $\beta$-gal assays or Y190 strain for HIS3 selection) containing either pAS.Gag (to confirm positive results), pVA3 (encoding p53), pLAM5′ (human lamin C), or the empty prey plasmid pAS2.1 as controls. Plasmids containing the cDNAs encoding fusion proteins that specifically interacted with Gag were isolated and sequenced. DNA sequence analysis and BLAST DNA and amino acid homology searches using this common open reading frame revealed that 15 of these clones were identical to human *ubc9*. cDNA clone G10B contained the entire, non-permuted *hUbc9* coding sequence and was chosen for further studies.

*Mammalian, in vitro, and bacterial expression plasmids*

For mammalian expression of Myc epitope-tagged hUbc9, the wild-type hUbc9 open reading frame, contained within an *Eco*RI-*Xho*I fragment from the cDNA clone pG10B, was subcloned into the *Eco*RI-*Sal*I restriction sites of pCMV.Tag 3A (Stratagene) to create pCMV.Myc-hUbc9. For bacterial expression of hUbc9–GST fusion proteins, the hUbc9 open reading frame in pCMV.Myc-hUbc9 was excised as a *Pst*I-*Xho*I fragment and subcloned into the corresponding sites of pET.41C(+) (Novagen) to create pET.Ubc9. The inserts of all newly constructed plasmid DNAs were sequenced to ensure that unwanted mutations were not inadvertently created. Expression of Myc–hUbc9 and hUbc9–GST fusion proteins in mammalian and bacterial cells, respectively, was confirmed by immunoblot analyses using anti-Myc, anti-GST, and anti-Ubc9 antibodies (data not shown).

*In vitro transcription/translation of MPMV Gag*

Transcription and translation reactions were performed simultaneously with pETM100A and the Coupled TNT Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

*Bacterial expression and purification of GST-fusion proteins*

*E. coli* BL21 (DE3) cells were transformed with either pET.Ubc9 or pET41c(+). Five hundred milliliter cultures of cells harboring either pET.Ubc9 or pET41C+ were grown to an optical density at 600 nm of 0.7, induced by the addition of 0.1 M isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) and incubated at 37°C for 3.5 h. Cells were chilled on ice, harvested by centrifugation at 6693 *g* for 15 min, and resuspended in 12.5 ml BugBuster reagent containing Benzonase Nuclease and rlysozyme (Novagen). Cells were lysed at room temperature for 20 min with gentle rocking. Cell debris was removed by centrifugation at 16,000 *g* (4°C) for 20 min. GST and hUbc9–GST fusion proteins were bound to glutathione–Sepharose (250 $\mu$l of settled resin per 500 ml culture; Novagen) for 8 h at 4°C with gentle rocking. Beads were washed with 20 column volumes of GBW buffer (4.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 275 mM NaCl, 2.7 mM KCl, pH 7.3) and eluted with 10 mM reduced glutathione (in 50 mM Tris–Cl, pH 8.0) according to manufacturer's suggestions. Reduced glutathione was removed by dialysis using three buffer (50 mM Tris–Cl, pH 8.0) changes. The purities and concentrations of the purified proteins were estimated by SDS–PAGE/Sypro orange (Bio-Rad Laboratory) staining and Lowry protein assays.

*GST pull-down assays*

To perform the GST pull-down experiments, approximately equal molar amounts of purified GST (12 $\mu$g) or hUbc9–GST (17 $\mu$g) were bound to 20 $\mu$l settled glutathione–Sepharose (Novagen) in 500 $\mu$l of GBW buffer. The resin was washed once with 1 ml GBW buffer and then incubated for 15 min with BSA (0.2 mg/ml) to block nonspecific protein-binding sites.

Reduced glutathione–Sepharose bound GST (12 $\mu$g) or Ubs9–GST (17 $\mu$g) fusion proteins were incubated (8 h at 4°C) with either pSARM4-transfected cell lysates, in vitro translation reactions containing Pr78, or 20 ng Gag-cleavage proteins obtained from purified MPMV virions that were solubilized in GTN buffer (GBW buffer, 275 mM NaC, 10.5% TX-100, 0.1% BSA). Beads were washed twice in GTN buffer and once in modified GBW buffer (same as GBW but with 135 mM NaCl). Bound proteins were eluted with protein sample buffer [10% glycerol, 2.3% SDS, 63 mM Tris–HCl (pH 6.8), 5% $\beta$-mercaptoethanol, 0.01% bromphenol blue] and separated by SDS–PAGE using either 12 or 15% acrylamide gels (Weldon et al., 1990). MPMV Gag proteins were analyzed by immunoblot analyses using polyclonal antibodies to Pr78, horseradish peroxidase (HRP) conjugated anti-rabbit antibodies, and Western Lighting (Perkin–Elmer) chemiluminescence reagent in accordance with manufacturer's suggestions. Polyclonal antisera against Pr78- and HRP-conjugated goat anti-rabbit immunoglobulin G were used at 1:10,000 and 1:30,000 dilutions, respectively. Blots were quantitated by densitometric analyses using a FluorS multi-imaging system and Quantity One software (Bio-Rad).

*Transfection and cell lysis*

CMMT cells and either COS-1 cells or HeLa cells (100-mm plates) were transfected with Fugene 6 and 2 $\mu$g pSARM4 (or pSHRM15). At 48 h posttransfection, media

were removed and cells were lysed on ice for 30 min with GTN buffer containing PMSF, leupeptin, pepstatin, and aprotinin. Cell lysates were clarified in a microcentrifuge for 2 min.

### *Coimmunoprecipitation of Gag and hUbc9–GST fusion proteins*

Uninfected HeLa cells, pSARM4-transfected HeLa cells, and MPMV virions (approximately 20 ng total Gag-related proteins) were lysed on ice for 30 min with GTN buffer. Samples were incubated with approximately equal molar amounts of soluble GST (12 $\mu$g) or hUbc9–GST (17 $\mu$g) proteins in GTN buffer containing 0.1% BSA. After incubation for 8 h at 4°C with gentle rocking, 1 $\mu$l of anti-Pr78 polyclonal antisera was added. Samples were incubated for an additional 4 h on ice. Antibody–antigen complexes were collected with formalin-fixed *saccharomyces aureus* cells (Sakalian et al., 1996a), washed twice in GTN buffer, once in 20 mM Tris–Cl (pH 6.3), separated by SDS–PAGE, and analyzed by immunoblot analyses using HRP-conjugated anti-GST antibodies (1:10,000 dilution).

### *Virus purification*

Tissue culture media were harvested from confluent monolayers of CMMT cells. After removing the cell debris by centrifugation at 6000 *g* for 10 min, virions were pelleted by centrifugation in a type 21 rotor (Beckman Instruments) at 21,000 rpm for 1 h at 4°C. The viral pellets were resuspended in PBS and layered onto continuous 20 to 60% (wt/wt) sucrose gradients in PBS. Gradients were centrifuged in a SW41 rotor (Beckman Instruments) for 16 h at 31,000 rpm at 4°C. Visible viral bands were collected, diluted fourfold in PBS, pelleted by centrifugation as above, and resuspended in PBS. Isopycnic-banded viruses were further purified by velocity sedimentation through continuous 5–20% (wt/wt) sucrose gradients by centrifugation in a SW41 rotor for 30 min at 31,000 rpm (4°C). Viral bands were collected, pelleted, and resuspended in PBS.

### *Subcellular localization*

HeLa and COS-1 cells were transfected for 48 h with pCMV.Myc-Ubc9 alone or in combination with pSARM4 using Fugene 6 (Boehringer Mannheim) according to manufacturer's suggestions. Cells were grown on coverslips, washed twice in PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and washed again in PBS. The cells were then permeabilized with 100% ethanol (−20°C) for 2 min, rehydrated with PBS, washed with PBS + 0.1% BSA, and incubated with primary antibodies, either a monoclonal anti-myc (1:60 dilution) and/or polyclonal anti-Pr78 (1:100 dilution), for 30 min at room temperature. Primary antibodies were detected using Cy2-labeled anti-rabbit IgG and Cy5-labeled anti-mouse IgG antibodies. Coverslips were mounted with Gel-mount and analyzed by confocal microscopy (Bio-Rad MRC 1024 ES microscope) using excitation wavelengths 506 nm (Cy2) and 670 nm (CY5). The channels were recorded separately and merged digitally.

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